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Correspondence and requests for materials should be addressed to T.M.C. (e-mail: tcronin@geochange.er.usgs.gov).

Evolutionary origin of insect wings from ancestral gills

Michalis Averof & Stephen M. Cohen

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

Two hypotheses have been proposed for the origin of insect wings. One holds that wings evolved by modification of limb branches that were already present in multibranched ancestral appendages and probably functioned as gills[1–5]. The second proposes that wings arose as novel outgrowths of the body wall, not directly related to any pre-existing limbs[6]. If wings derive from dorsal structures of multibranched appendages, we expect that some of their distinctive features will have been built on genetic functions that were already present in the structural progenitors of insect wings, and in homologous structures of other arthropod limbs. We have isolated crustacean homologues of two genes that have wing-specific functions in insects, *pdm* (*nubbin*) and *apterous*. Their expression patterns support the hypothesis that insect wings evolved from gill-like appendages that were already present in the aquatic ancestors of both crustaceans and insects.

Developmental studies have shown that wings and legs originate in a common primordium in early insect embryos[7] and that they share a common regulatory mechanism for patterning along the antero–posterior (AP) axis[8], based on the contiguous domain of *engrailed* (*en*) expression at the posterior of each segment. Crustaceans appear to be the closest living relatives of insects that retain a primitively multibranched structure in their limbs[9,10], and these limbs show comparable expression of *engrailed*, in a contiguous domain that runs along the posterior of each limb branch (N. Patel and M.A., manuscript in preparation; Fig. 1). Thus, the embryonic origin and relative organization of insect legs and wings appear comparable to those of ventral and dorsal branches of crustacean limbs. Although AP patterning appears to be similar in legs and wings, several genes, including *vestigial*, *scalloped*, *wingless*, *pdm* and *apterous*, have been identified with distinctive roles in establishing the wing primordiun and in patterning the wing along the dorso–ventral (DV) axis[11–17] (Fig. 1).

To determine whether genes with wing-specific functions in insects might play a specific role in patterning dorsal elements of multibranched crustacean appendages, we isolated homologues of the *Drosophila* genes *pdm* and *apterous* (*ap*) from the branchiopod crustacean *Artemia franciscana* (named *Af-pdm* and *Af-ap*, respectively). Amino-acid sequence comparison reveals a high degree of conservation within regions of known sequence motifs and indicates that these are orthologues of the *Drosophila pdm* and *apterous* genes (Fig. 2). In *Drosophila* there are two closely related *pdm* genes, *pdm1* and *pdm2*, with largely overlapping expression patterns and functions[18–20]. We have recovered a single *pdm* gene in *Artemia* (7/7 sequenced clones) with sequence that is approximately equidistant from *pdm1* and *pdm2* (Fig. 2b). In view of the close functional relationship of the two *Drosophila pdm* genes[19,20], we suspect that *Af-pdm* may be functionally equivalent to both.

One of the *Drosophila pdm* genes, *pdm1* (*nubbin*), is expressed throughout the prospective wing and has been implicated in the early specification of the wing primordium[13,14]. It is also expressed more weakly in a set of rings in the primordia of legs, where its function is unknown[13]. To compare the expression of *pdm* in *Artemia* with that seen in *Drosophila*, we have raised antibodies against Af-PDM. Af-PDM is expressed in a dynamic pattern in the developing thoracic limbs (Fig. 3c–e). At early stages, the gene is expressed over most of the developing limb bud. However, as soon as the appendage shows the first signs of regionalization, this expression becomes restricted to a dorsal lobe that will give rise to the distal epipodite. During subsequent stages, expression is maintained specifically in the distal epipodite.

In insects, *apterous* is expressed specifically on the dorsal surface of developing wings[21,22] and appears to be the primary determinant for DV patterning of wings[11,16,17]. It is also expressed in a ring in the fourth tarsal segment of *Drosophila* legs, although this expression appears to have no function[21]. Using antibodies raised against Af-AP, we find that this protein is expressed in a pattern similar to that of Af-PDM, with expression in the early limb primordium becoming restricted to the distal epipodite at the time when this structure first becomes morphologically distinct (Fig. 3f–h). Expression is not restricted to the dorsal aspect of this branch; this is not surprising as this lobe shows no sign of dorso–ventral differentiation in its morphology. In addition, at late stages Af-AP expression appears in the limb muscles.

It is of interest that Af-PDM and Af-AP become specifically expressed in the cells of the distal epipodite before these acquire their characteristic differentiated morphology (large nuclei, large intercellular spaces). These expression patterns contrast markedly with that of *Distal-less* (*Dll*), which is expressed in all outgrowing appendages (including insect legs and wings, and all crustacean limb branches[23,24]; Fig. 3b).

One of our antibody preparations, raised against Af-PDM, fortuitously recognizes a conserved epitope in PDM proteins of

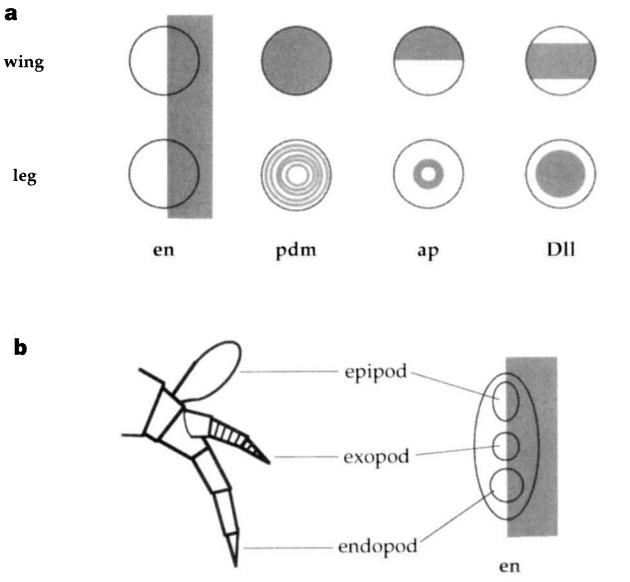

Figure 1 a, Representation of insect legs and wings, indicating the expression of *engrailed* (*en*), *nubbin/pdm*, *apterous* (*ap*), and *Distal-less* (*Dll*) (based on refs 13, 26). The expression of *ap* in the leg appears to have no function[21]. Orientation: dorsal is up, anterior is to the left, and the proximo-distal axis runs perpendicular to the page, with distal regions represented at the centre and proximal regions (attachment to the body wall) at the periphery of each disc. **b**, The multibranched structure of a diagrammatic crustacean limb, indicating its anteroposterior organization relative to the expression of *engrailed* (N. Patel and M.A., manuscript in preparation). The drawing showing *engrailed* expression is oriented as in **a**.

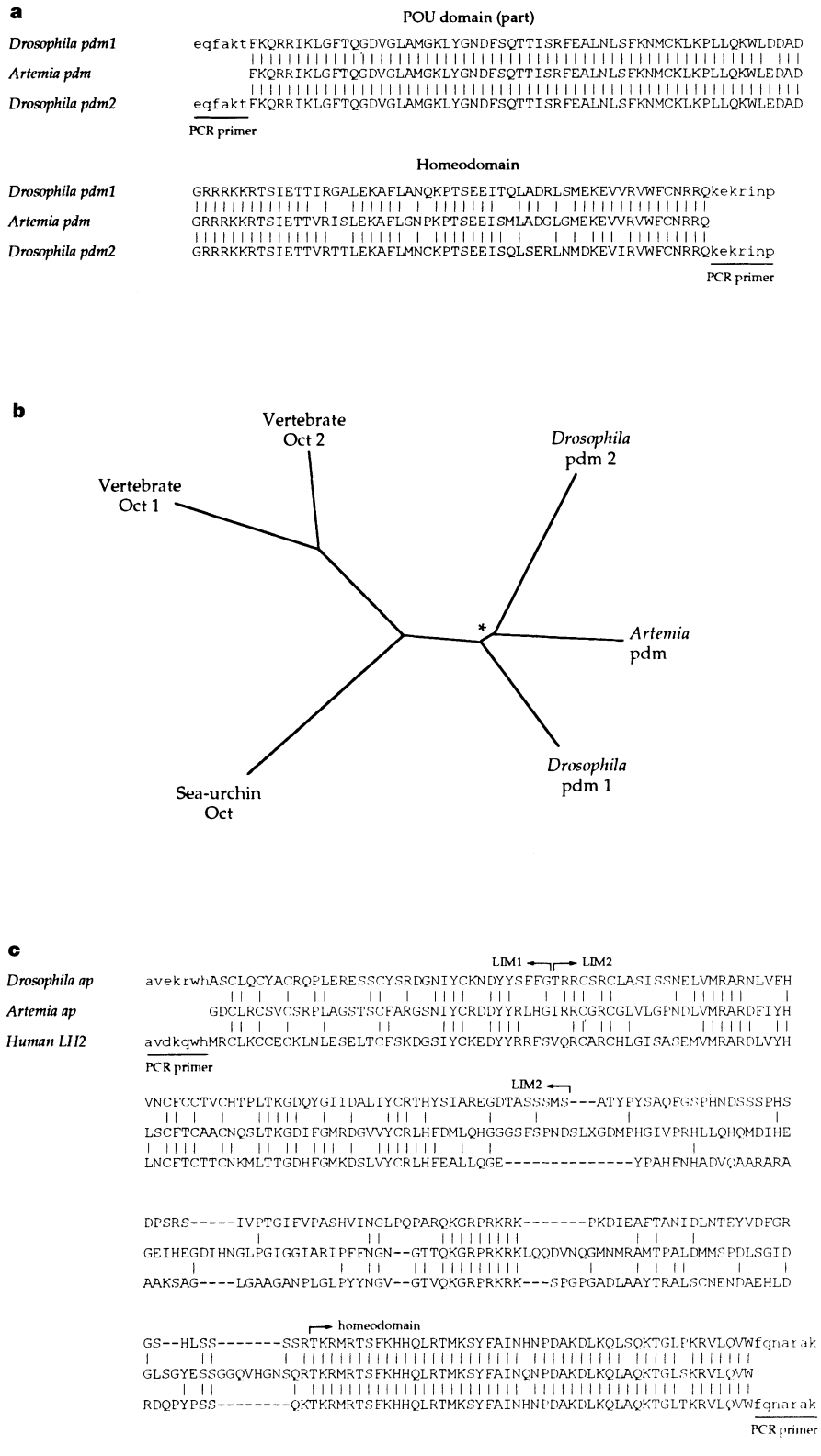

Figure 2 **a**, Sequence alignment of Af-PDM with its *Drosophila* counterparts Pdm1 and Pdm2. Alignments are shown for the POU domain and homeodomain regions only; no significant sequence similarity was found outside these regions. Amino-acid identity is indicated by a vertical line. The positions of PCR primers used for the isolation of the *Artemia* gene (see Methods) are shown. **b**, Tree depicting the sequence relationships of *Af-pdm* with *Drosophila pdm1* and *pdm2*, and their counterparts from vertebrates and sea urchins (Oct genes). Comparisons were made at the amino-acid level on the available POU and homeodomain sequences only (as in **a**). The node between *Af-pdm*, *pdm1* and *pdm2* (marked by an asterisk) is not significantly resolved. **c**, Sequence alignment of Af-AP with its *Drosophila* and vertebrate counterparts AP and LH2. The entire region available for Af-AP is shown, including regions of the two LIM domains (part of Lim1 and Lim2) and part of the homeodomain. Amino-acid identity and the position of PCR primers are indicated as in **a**. Sequence alignments and the tree were prepared using the CLUSTAL V program[27]. Sequence accession numbers: *Af-pdm*, Y09913; *pdm1*, M81957; *pdm2*, M81958; *Oct1*, X13403; *Oct2*, M36653; *Sp-Oct*, L04646; *Af-ap*, Y09914; *ap*, X65158; *LH2*, U11701.

other species (data from *Drosophila*, not shown). We have been able to use this antibody to study *pdm* gene expression in the crayfish *Pacifastacus leniusculus*. In the thoracic limbs of this species, we observe two distinctive patterns of *pdm* expression (Fig. 3i–k): expression throughout a distal epipodite lobe (similar to that seen in *Artemia*) and additional expression in a set of rings along the leg, reminiscent of *pdm* expression in insect legs[13] (Fig. 1a). *pdm* expression is absent from the endopods of the first thoracic segment in crayfish (Fig. 3i), which do not develop the characteristic articulated morphology of legs, as in *Artemia* limbs (Fig. 3d, e).

We have reasoned that the relationship of insect wings to other structures may be revealed by commonly inherited patterns of gene expression. In this respect, some genes are likely to be more informative than others. Genes like *Dll* and *engrailed* are unlikely to be informative because they are expressed in similar patterns in all outgrowths from the body wall (refs 24, 25, and N. Patel and M.A., manuscript in preparation), and could have been co-opted into patterning of wings irrespective of their origin. *pdm* and *apterous* appear to be more suitable markers as they have important functions that are more specific to particular appendages. Our observations in *Artemia* indicate that the activities of both *pdm* and *apterous* are associated specifically with a distal epipodite of crustacean limbs. Moreover, we have shown that *pdm* expression is conserved in representatives of two major divergent clades of crustaceans with very different limb architectures, branchiopods and malacostracans. Crustacean epipodites are dorsally located limb branches with respiratory and osmoregulatory functions, precisely the type of structure that, according to some hypotheses, would

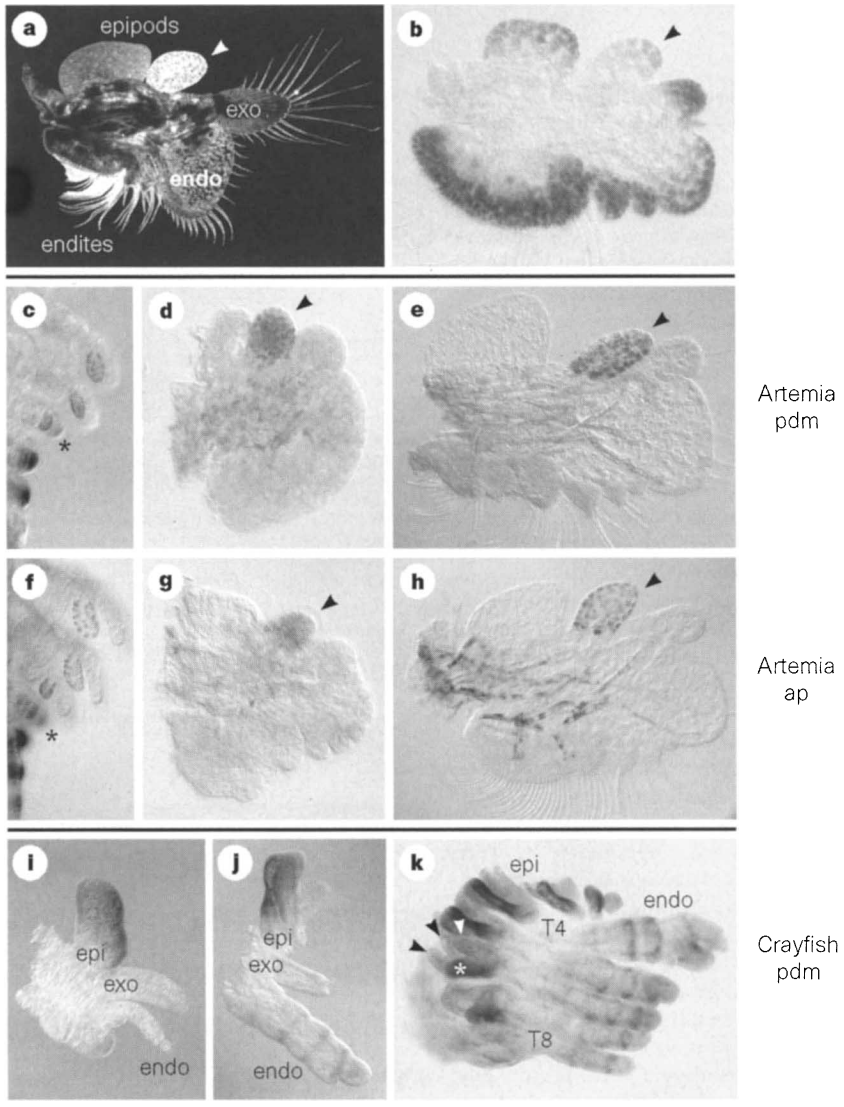

Figure 3 Expression of PDM and AP in developing crustacean appendages. **a**, The branched morphology of an *Artemia* thoracic limb, indicating the position of the two epipodites, the exopod, endopod and endites. Dorsal is up, proximal (attachment to the body wall) is to the left. **b**, Expression of DLL in all outgrowing regions of the *Artemia* limb[24]. **c**–**e**, Expression of Af-PDM. **f**–**h**, Expression of Af-AP. **c** and **f**, Dorsal view of a series thoracic appendages in the body of a developing *Artemia* larva. Posterior segments (at the bottom) carry younger limb buds, whereas more anterior appendages (towards the top) represent progressively more mature stages. The stage at which expression becomes restricted to the distal epipodite is marked by an asterisk. **d**, **e** and **g**, **h**, Individual dissected limbs oriented as in **a**. In **d** and **g**, In young limb buds, soon after the earliest regional features (distinct lobes) have become visible. These limbs are of comparable age to those marked by asterisks in **c** and **f**, **e** and **h**. Later, more mature limbs. Arrowheads mark the distal epipodite. **i**–**k**, PDM expression in the thoracic limbs of *Pacifastacus leniusculus* at ~70% of embryonic development; **i**, limb of first thoracic segment (T1); **j**, limb of third thoracic segment (T3); **k**, limbs of thoracic segments 4–8 (T4–T8), epipodites of T2 and T3 are also visible anteriorly. Limbs in **i** and **j** are oriented with dorsal side up and proximal towards the left; limbs in **k** are viewed from the dorsal side, oriented with anterior limbs towards the top and proximal parts towards the left. Epipodites, exopods and endopods are labelled where present (exopods absent from T4–T8). Strong staining can be seen in a single, distal and posterior epipodite lobe per limb (lacking in T8) marked by an asterisk in T6 of panel **k**; other epipodite lobes on the same limb are marked by arrowheads.

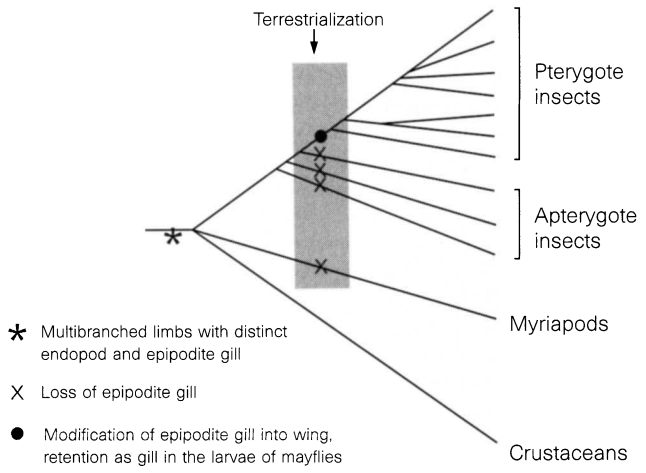

Figure 4 The phylogenetic distribution of respiratory epipodites, wings and gills. Respiratory epipodites are inferred to have been present in the last common ancestor of crustaceans, insects, and possibly myriapods. Thus, the ancestors of insects are likely to have been aquatic animals bearing multibranched appendages with distinct locomotory and respiratory parts (legs and epipodite gills). During the transition from aquatic to terrestrial life epipodite gills must have gradually lost their utility as respiratory and osmoregulatory organs. As a result, gills appear to have been lost independently in myriapods and most of the early lineages of insects (apterygotes). In the lineage that gave rise to the winged insects (pterygotes), we propose that these structures were retained in modified form, perhaps initially as gills in aquatic larvae (as in the abdominal segments of present-day mayfly larvae) and subsequently as wings. The relationships between crustaceans, myriapods and insects remain controversial[9,10] and are therefore presented as an unresolved trichotomy.

have given rise to the insect wings[1–5]. We therefore support the idea that insect wings have evolved from gill-like appendages, and propose that they may be homologous to specific epipodites of crustacean limbs.

Homology of divergent structures can never be proven with certainty. Arguments based on gene expression must take into account that individual genes can acquire different roles in different developmental contexts. For example, both *pdm* and *apterous* show distinctive patterns of expression in wings and legs in *Drosophila*[13,21]. In the case of *pdm*, we have shown that similar distinctive patterns of expression exist in the epipodites and legs (respectively) of crustacean limbs, when these structures exist. Thus we suggest that distinct structural progenitors of legs and epipodites/wings were present in the last common ancestor of crustaceans and insects (Fig. 4). We take this as evidence for a direct evolutionary relationship between epipodites and wings. An alternative interpretation might be that wings do not derive from epipodites but have nevertheless independently coopted a number of gene functions that were already used in epipodites. Although formally possible, we consider this less likely.

The proposed evolution of wings from epipodites presumably involved several genetic and developmental changes, required for repatterning of these structures. At the genetic level, these changes must have utilized and modified gene functions that were already operating in respiratory epipodites. One example may be the use of *apterous* expression and its restriction to the dorsal surface of the wing to initiate a DV patterning system not used in the ancestral limb or in present-day insect legs[16,17]. A second modification must have resulted in the physical separation of the epipodite from the leg, perhaps by fusion of the limb base with the body wall[3,5]. □

Methods

Af-pdm and *Af-ap* were cloned by polymerase chain reaction (PCR) using degenerate primers. Primers were 5′-GGAATTC GA(A/G) CA(A/G) TT(T/C) GCI AA(A/G) AC-3′ and 5′-GCTCTAGA GG(A/G)TT IAT IC(T/G) (T/C)TT (T/C)TC (T/C)TT-3′ for *Af-pdm*, and 5′-GGAATTC GCI GTI GAI AA(A/G) C(A/G)I TGG CA-3′ and 5′-GCTCTAGA TT IGC IC(T/G) IGC (A/G)TT(T/C)TG (A/G)AA-3′ for *Af-ap* (where I is inosine). PCRs were carried out on first-strand cDNA prepared from early larval stages of *Artemia franciscana*; 7 and 4 independent clones were sequenced for *Af-pdm* and *Af-ap* respectively. *Af-pdm* and *Af-ap* were subcloned into expression vectors pATH-1 and pET-23a and expressed in bacteria. Antibodies against Af-PDM and Af-AP were raised in mice by repeated injection of 70-μg doses of bacterially expressed protein in Freund's adjuvant. Immunochemical staining was done using 1:500 dilutions of mouse serum. *Artemia* larvae were sonicated before staining to allow efficient penetration of reagents. The DLL staining in Fig. 3b was done with the antibody described in ref. 24. Detailed protocols are available upon request.

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Correspondence and requests for materials should be addressed to M.A. (averof@embl-heidelberg.de). The *Af-pdm* and *Af-ap* sequences have been deposited in the EMBL database under accession numbers Y09913 and Y09914.

Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors

Massimo Scanziani*†, Paul A. Salin*†, Kaspar E. Vogt*, Robert C. Malenka‡§ & Roger A. Nicoll*‡

*Departments of * Cellular and Molecular Pharmacology, ‡ Physiology, and § Psychiatry, University of California at San Francisco, San Francisco, California 94143, USA*

The classical view of fast chemical synaptic transmission is that released neurotransmitter acts locally on postsynaptic receptors and is cleared from the synaptic cleft within a few milliseconds by

† Present addresses: Laboratoire de Physiologie, Ecole Superieure de Physique et Chimie Industrielles, 10 rue Vauqeulin, 75005 Paris, (M. S.); Institut Alfred Fessard, Av. de la Terrasse, 91198 Gif sur Yvette cedex, France (P. A. S.).